Structure of Polyubiquitinated Histone H2A†

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ABSTRACT: We have recently demonstrated that trout liver histones H2A, H2B, and H2A.Z can be polyubiquitinated [Davie, J. R., Delcuve, G. P., Nickel, B. E., Moyer, R., & Bailey, G. (1987) *Cancer Res. 47*, 5407-5410]. In the present study we determined the arrangement of the ubiquitin molecules in polyubiquitinated histone H2A. Trout liver chromatin fragments, which had histone H1 removed, were digested with *Staphylococcus aureus* (V8 strain) protease which cleaves specifically on the carboxyl side of glutamic acid residues under the conditions used. The V8 protease readily degraded histone H2A and ubiquitinated (u) H2A at equivalent rates. One site in H2A and uH2A, the peptide bond between Glu 121 and Lys 122, was cleaved, yielding protein species cH2A and cuH2A, respectively. None of the other nucleosomal histones (H2B, H2A.Z, H3, and H4) including uH2B and uH2A.Z were sensitive to digestion. Trout liver histones cleaved with either V8 protease, histone H2A specific protease, or cyanogen bromide were resolved by two-dimensional gel electrophoresis and ubiquitinated peptides detected with anti-ubiquitin IgG. The results suggest that the major arrangement of ubiquitin in polyubiquitinated H2A is a chain of ubiquitin molecules joined to each other by isopeptide bonds to a ubiquitin molecule that is attached to the ϵ-amino group of lysine 119 of histone H2A.

In the nucleus ubiquitin, a 76-residue protein can be found covalently joined via an isopeptide linkage to approximately 10% of the nucleosomal histone H2A (Busch & Goldknopf, 1981) and to approximately 1-2% of the histone H2B (West & Bonner, 1980). Ubiquitinated forms of histone H2A and histone H2B are known as uH2A and uH2B, respectively. In calf thymus uH2A the C-terminal glycine of ubiquitin is attached to the ϵ-amino group of lysine 119 in the C-terminal portion of the histone H2A molecule (Goldknopf & Busch, 1977). The site of ubiquitin attachment in uH2B from calf and pig is identical, with an isopeptide bond formed between the C-terminal glycine of ubiquitin and the ϵ-amino group of lysine 120 (Thorne et al., 1987).

Our observations of polyubiquitinated histones in trout testis and trout liver chromatin were the first reports of naturally occurring histones with more than one ubiquitin attached (Nickel et al., 1987; Davie et al., 1987). In this study the arrangement of ubiquitin molecules in polyubiquitinated H2A from trout liver was determined. The attachment of several ubiquitin molecules to one protein molecule can theoretically occur in more than one way. Figure 1 illustrates four possible models for the arrangement of more than one ubiquitin molecule on the histone H2A molecule. A combination of these models may also be possible. In the first model additional ubiquitin molecules are linked through their C-terminal glycine residues to ϵ-amino groups of lysine residues distributed along the histone H2A molecule. The remaining models show additional ubiquitin linked to ubiquitin molecules already joined to histone H2A.

Our strategy to determine the arrangement of ubiquitin molecules in ubiquitinated histone H2A was to cleave trout liver histones with either V8 protease or histone H2A specific protease (Figure 2). H2A-specific protease acts exclusively on histone H2A and its ubiquitinated forms and catalyzes the removal of 13 amino acids from the carboxyl end of the H2A molecule by specific cleavage between valine 114 and leucine 115 (Eickbush et al., 1976; Watson & Moudrianakis, 1982; Davie et al., 1986). Digestion of chromatin with *Staphylococcus aureus* (V8 strain) protease results in cleavage at the carboxyl side of glutamic acid 121 of histone H2A and its ubiquitinated species. Ubiquitin itself is not digested by these enzymes. The sites of cleavage of V8 protease and histone H2A specific protease set boundaries around a seven amino acid region of the H2A molecule that contains two lysine residues at positions 118 and 119 (see Figure 2). If the site of ubiquitin attachment is conserved in uH2A, then ubiquitin will be attached to Lys 119 of trout histone H2A. Peptide products of protease digestions were resolved by two-dimensional gel electrophoresis and the ubiquitinated peptides on Western blots detected immunochemically with anti-ubiquitin IgG and ^{125}I-labeled protein A. Our results demonstrate that the seven-residue fragment of H2A can contain at least three ubiquitin molecules.

Materials and Methods

Preparation of Trout Liver Histones and Chromatin. Liver nuclei isolated as described previously (Nickel et al., 1987) were resuspended to a concentration of 40 A_{260} units/mL in buffer B containing 1 mM $CaCl_2$. Following preincubation at 37 °C, 50 A_{260} enzyme units/mL of micrococcal nuclease were added and the nuclei digested for 10 min at 37 °C. Digestion was terminated by placing the suspension on ice and adding EGTA to 10 mM. Digested nuclei, which were collected by centrifugation at 2000g for 10 min, were resuspended in 1 mM EDTA, pH 7.5, and 1 mM PMSF. This results in solubilization of the majority of the chromatin. The insoluble material was removed by centrifugation at 12000g for 10 min, yielding supernatant fraction S_{EDTA}. Acid-soluble proteins were extracted from the supernatant with 0.4 N H_2SO_4. In addition H1-stripped chromatin was prepared. The supernatant (S_{EDTA}) was adjusted to 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 10 mM EDTA, and CM-sephadex was added as described by Libertini and Small (1980). The suspension was filtered and dialyzed against 10 mM EDTA, pH 7.5, prior

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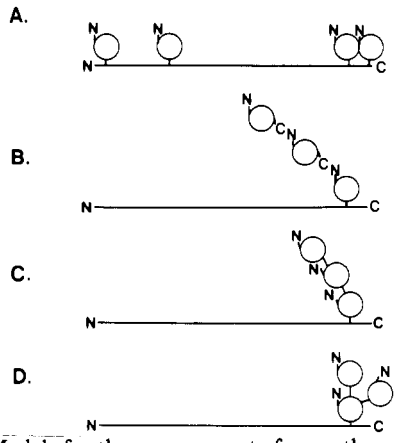

FIGURE 1: Models for the arrangement of more than one ubiquitin molecule on the histone H2A molecule. Histone H2A is shown as a straight line, and ubiquitin molecules are shown as circles. Models are described in the text.

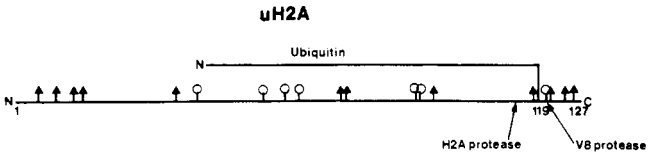

FIGURE 2: Schematic illustration of the trout uH2A molecule. The attachment of ubiquitin to histone H2A at lysine 119 is shown as well as the location of additional lysine residues (↑) and glutamic residues (♀). The sites for cleavage by H2A-specific protease and V8 protease are indicated.

to concentration against polyethylene glycol (PEG). This procedure removes histone H1 and the majority of the nonhistone chromosomal proteins.

Cleavage of Histones with Cyanogen Bromide. Cleavage of acid-extracted proteins with cyanogen bromide was done as described by Tung et al. (1984). Briefly, 60 μg of acid-extracted protein was resuspended in 100 μL of 44% formic acid. A small piece of cyanogen bromide (CNBr; approximately 1 mg) was added to the tube. A control sample in 44% formic acid but without CNBr was also prepared. The samples were incubated 16 h at 4 °C and lyophilized. The proteins were resuspended in 6 μL of double-distilled H_2O (ddH_2O) and 4 μL of reducing solution [1:1:1:1 v/v ddH_2O, 1 M Tris–acetate, pH 8.8, 2-mercaptoethanol, and 20% (w/v) cysteamine], mixed with 10 μL of AUT sample buffer [8 M urea, 0.75 M potassium acetate, pH 4.0, 30% (w/v) sucrose, and 0.1% (w/v) pyronin Y]. Proteins were resolved by two-dimensional gel electrophoresis [acetic acid/6.7 M urea/0.375% (w/v) Triton X-100 (AUT), 15% polyacrylamide gel electrophoresis in the first dimension and sodium dodecyl sulfate (SDS) 15% polyacrylamide gel electrophoresis in the second]. Proteins were electrophoretically transferred to nitrocellulose and immunochemically stained for ubiquitin with an anti-ubiquitin IgG and ^{125}I-labeled protein A as described by Nickel et al. (1987).

S. aureus (V8 Strain) Protease Digestion of Chromatin. Chromatin (stripped of histone H1) at 1 mg/mL in 50 mM NH_4HCO_3, pH 8.0, and 0.2 mM EDTA was incubated with or without *S. aureus* protease (V8 strain, Miles Scientific) at 0.25 mg/mL (protease/chromatin = 25 μg/100 μg) at 37 °C for 1, 2, or 3 h. The reaction was stopped by placing the samples on ice, adding $^1/_{10}$ volume of 8.3 N NH_4OH, heating the sample at 100 °C for 1 min, cooling on ice, and adding PMSF to 10 mM. The sample was lyophilized and prepared for AUT gel electrophoresis by protamine release (Nickel et al., 1987). Proteins were resolved by two-dimensional gel electrophoresis (AUT into SDS), electrophoretically transferred to nitrocellulose, and immunochemically stained for ubiquitin. The extent of H2A digestion was determined by removing a portion of the sample following incubation, adding it directly to an equal volume of SDS sample buffer (Davie, 1982), heating to 100 °C for 1 min, and analyzing the proteins by SDS gel electrophoresis. Gels were stained with Coomassie blue or silver (Nickel et al., 1987).

H2A-Specific Protease Digestion of Histones. An enriched preparation of H2A specific protease was obtained from calf liver chromatin. Calf liver nuclei were isolated as described previously (Davie et al., 1986) and resuspended at 40 A_{260} units/mL in buffer B containing 1 mM $CaCl_2$. Nuclei were digested with 50 A_{260} enzyme units/mL of micrococcal nuclease at 37 °C for 20 min. The reaction was terminated by placing the suspension on ice and adding 0.25 M EGTA to a final concentration of 10 mM. The digested nuclei were collected by centrifugation at 750*g* for 10 min and then resuspended in 10 mM EDTA. The insoluble nuclear material was removed by centrifugation at 12000*g* for 10 min. The resulting supernatant was adjusted to 150 mM NaCl by the dropwise addition of 2 M NaCl and centrifuged at 12000*g* for 10 min, and the pellet material was saved. H2A-specific protease was separated from the bulk of the chromatin by gel exclusion chromatography on a Bio-Gel A-5m column (30 × 1.5 cm). The pellet was redissolved at approximately 50–70 A_{260} units/mL in the column elution buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 4 M NaCl) and approximately 0.5 mL loaded onto the column. Two-milliliter fractions were collected, and the elution of chromatin was monitored at 260 nm. Column fractions were assayed for H2A-specific protease activity according to the method of Davie et al. (1986). Briefly, 20 μL of calf thymus histone H2A (10 mg/mL) was mixed with 230 μL of a column fraction, and 250 μL of 20 mM Tris, pH 10.0, was added, resulting in a pH of 8.5. Samples were incubated at 37 °C for about 2 h. Following incubation, 1.5 mL of ddH_2O was added per sample, and the proteins were acid-extracted with 0.4 N H_2SO_4. The percentage of H2A molecules cleaved was determined as described by Eickbush et al. (1976) by scanning Coomassie blue stained 15% polyacrylamide SDS gels. Fractions with protease activity were pooled, concentrated against PEG, and used as the source of H2A-specific protease for further studies. It was possible to store the concentrated enzyme fraction for at least two weeks without significant loss of activity. One milligram of BSA was added for each milliliter of enzyme preparation, and aliquots were stored at −80 °C.

To digest trout liver histones, 200 μg of histones was mixed with ddH_2O and the H2A-specific protease preparation to give a final volume of 250 μL. An equal volume of 20 mM Tris, pH 10.0, was added, and the samples were incubated at 37 °C for 2 and 4 h. Following digestion 1.5 mL of ddH_2O was added per sample, and the proteins were acid-extracted. Experiments were done where only the enzyme preparation was added. Immunochemical staining of these Western blots demonstrated that the enzyme preparations had undetectable to low levels of ubiquitinated histones.

RESULTS

The Carboxyl-Terminal Portion of Histone H2A and Ubiquitinated H2A in Trout Liver Chromatin Is Readily Digested by S. aureus Protease. Trout liver chromatin stripped of histone H1 and nonhistone chromosomal proteins was digested with V8 protease (*S. aureus* protease, V8 strain) (Figure 3). V8 protease has been used as a probe of exposed nonbasic residues in nucleosomes (Rill & Oosterhof, 1981).

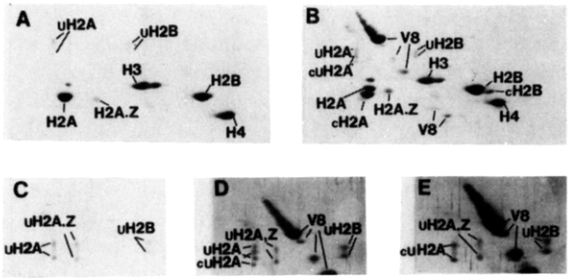

FIGURE 3: V8 protease selectively digests H2A and uH2A in trout liver chromatin. Chromatin isolated from trout liver was incubated without (A, C) or with (B, D, E) V8 protease at 37 °C for 1 h (B, D) or 3 h (E). The histones were resolved by two-dimensional gel electrophoresis (AUT into SDS). The gels were stained with Coomassie blue. The gels shown in (C)–(E) show a portion of the gel pattern stained with silver. cH2A, cuH2A, and cH2B denote cleaved forms of the H2A, uH2A, and H2B, respectively. V8 represents the intact protease and its proteolytic forms. Note that on SDS gels the ubiquitinated histone species migrate as doublets.

In the absence of exogenous protease, the histones in trout liver chromatin remained intact during a 3-h incubation period at 37 °C. In the presence of V8 protease, histone H2A was the most vulnerable histone in chromatin to protease attack. Approximately 50% of histone H2A was cleaved after 1 h of incubation (Figure 3), and the majority of histone H2A was digested after 3 h. The size of the cleaved H2A (cH2A) indicates that the V8 protease attacked the peptide bond on the carboxy side of Glu 121, which would result in the removal of six residues from the carboxyl-terminal portion of histone H2A (Connor et al., 1984). Inspection of the two-dimensional gel pattern demonstrates that cH2A migrates slightly slower than H2A on AUT gels, indicating that the removal of six C-terminal amino acid residues does not affect the extent of Triton X-100 binding. As three of these six amino acid residues are lysines, this may account for the slower cathodal migration of cH2A on AUT gels.

Histone H2B showed a slight sensitivity to protease attack. The identification of the cleaved form of H2B (cH2B) was confirmed by comparative peptide mapping on SDS–polyacrylamide gels (not shown). Neither histone H2A.Z, H3, nor H4 was noticeably sensitive to proteolysis.

The susceptibilities of the ubiquitinated histones of H2A, H2A.Z, and H2B in liver chromatin to digestion by V8 protease are shown in Figure 3. Note that on SDS gels the ubiquitinated histone species migrate as doublets (Davie et al., 1986). A possible explanation for this anomalous migration is that on AUT gels, which contain 6.7 M urea, the ubiquitin molecule is fully denatured, but when electrophoresed into a second-dimension SDS gel, ubiquitin can refold or remain fully denatured, generating two forms of the same protein–ubiquitin conjugate. The upper band of the doublet is presumably the fully denatured ubiquitin–histone conjugate. We have observed that the relative proportion of the two bands is variable from gel to gel; for example, see Figure 6, where the lower band of uH2A.Z predominates in the control sample but the upper band is the major form in the CNBr-treated sample.

Purified ubiquitin was not digested by V8 protease (not shown), in agreement with the protease-resistant properties of ubiquitin (Vijay-Kumar et al., 1985). Thus, if a ubiquitin–histone conjugate was cleaved, cleavage would be in the histone portion of the conjugate. Of the three ubiquitinated histone species, only uH2A was digested (Figure 3). After 1 h of incubation, two doublets (uH2A and cuH2A) were observed, and after 3 h, only the faster migrating doublet (cuH2A) remained, indicating that ubiquitin remains attached to cuH2A. The rate of conversion of uH2A to cuH2A appears to be similar to that for H2A to cH2A; i.e., after 1 h, approximately 50% of H2A and uH2A were converted to their cleaved forms.

Ubiquitin Is Not Attached to H2A between Lys 122 and the C-Terminal Lys 127. Ubiquitinated histone conjugates resolved by two-dimensional gel electrophoresis were detected on Western blots by immunochemical staining with anti-ubiquitin IgG and ^{125}I-labeled protein A (Figure 4). Trout liver histones H2A, H2B, and H2A.Z can be polyubiquitinated, with histone H2A having the greatest level of polyubiquitinated species. When the log molecular weight of the ubiquitinated H2A species is plotted versus the mobility on SDS 15% polyacrylamide gels, the points lay on a straight line (Figure 4C).

Digestion of trout liver chromatin with V8 protease converts the ubiquitinated H2A species (uH2A, u_2H2A, u_3H2A, u_4H2A) to slightly faster migrating forms (cuH2A, cu_2H2A, cu_3H2A, cu_4H2A) that correspond to ubiquitinated species of cleaved histone H2A that have lost six C-terminal amino acid residues (Figure 4). This is seen by comparing the mobility of the digested and undigested ubiquitinated H2A species to either the ubiquitinated H2B or ubiquitinated histone H2A.Z species that are not digested by V8 protease (Figure 4C). Ubiquitin is not attached to the six-residue fragment

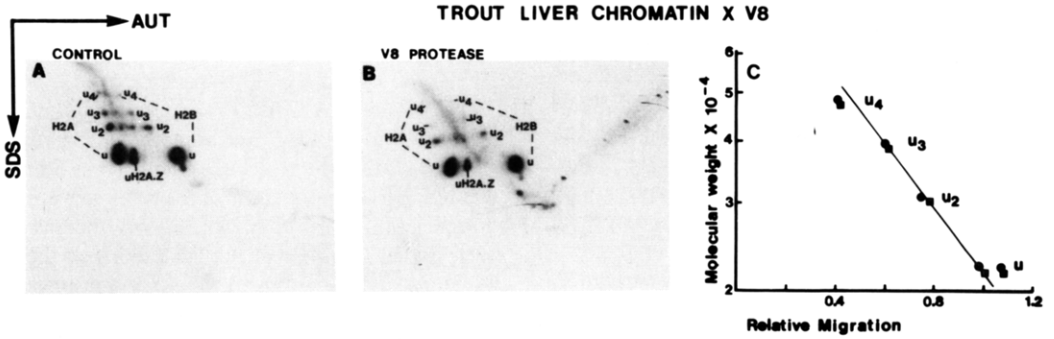

FIGURE 4: Identification of ubiquitinated peptides following digestion of trout liver chromatin by V8 protease. Trout liver chromatin with histone H1 removed was incubated without (A) or with (B) V8 protease, and the resulting peptides were resolved by two-dimensional gel electrophoresis (AUT into SDS). Peptides were electrophoretically transferred to nitrocellulose and immunochemically stained for ubiquitin with anti-ubiquitin IgG and ^{125}I-labeled protein A. The autoradiograms are shown. The ubiquitin adducts of histones H2A, H2A.Z, and H2B are denoted uH2A, uH2A.Z, and uH2B, respectively. The polyubiquitinated histone species are labeled u_2, u_3, and u_4, representing the attachment of two, three, or four ubiquitins, respectively. The spots to the right side of uH2B and the spot below uH2B, which are present in panel B but not in panel A, are staining artifacts. (C) The migration on the second-dimension SDS gel of each ubiquitinated histone H2A species (cleaved, ■, and uncleaved, ●) relative to that of uH2A.Z (upper band) was plotted against the estimated molecular weight of each form. The molecular weights of histone H2A and the N-terminal H2A peptide (1–121) were assumed to be 14 000 and 13 300, respectively. Each ubiquitin moiety would increase this molecular weight by 8500. Monoubiquitinated H2A and cH2A gives two species on SDS gels.

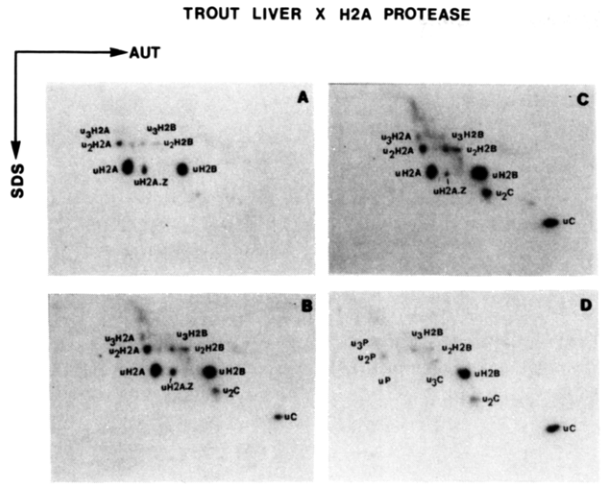

FIGURE 5: Identification of ubiquitinated peptides following digestion of trout liver histones by H2A specific protease. Trout liver histones were digested with an enriched preparation of calf liver H2A specific protease. The resulting peptides were analyzed as described in Figure 4. Ubiquitinated fragments of the H2A molecules are designated uC, u_2C, and u_3C if they contain the C-terminal portion (115–127) of the H2A molecule and uP, u_2P, and u_3P if they contain a larger fragment of the H2A molecule. (A) Undigested trout liver histones; (B) trout liver histones digested 2 h with 130 μL of H2A protease preparation; (C) trout liver histones digested 4 h with 130 μL of H2A protease preparation; (D) trout liver histones digested 4 h with 180 μL of H2A protease preparation.

(122–127) of histone H2A, as an additional ubiquitinated peptide of approximately 9.1 kDa is not seen. (This peptide would be located in a position similar to that of uC shown in Figure 5B.) Moreover, free ubiquitin was not detected, suggesting that V8 protease does not release ubiquitin from polyubiquitinated histone species.

Ubiquitin Is Principally Attached to Histone H2A between Leu 115 and the C-Terminal Lys 127. Histone H2A specific protease was partially purified from calf liver chromatin, which is a rich source of this enzyme (see Materials and Methods; Davie et al., 1986). Neither trout liver nor trout testis chromatin contains this protease activity (Davie, unpublished observations). Digestion of trout liver histones with histone H2A specific protease results in the appearance of two ubiquitinated peptides, uC and u_2C. The mobility of these ubiquitinated peptides on the SDS–polyacrylamide gel was consistent with a 13 amino acid histone H2A peptide (115–127; molecular weight 1438) with one or two ubiquitin molecules attached, having molecular weights of approximately 9.9 and 18.4, respectively (Figure 5). If one of the ubiquitin molecules in diubiquitinated and/or triubiquitinated H2A was attached on the N-terminal side of the scissile bond (i.e., the bond between Val 114 and Leu 115), then we would expect to see the generation of a ubiquitinated peptide that migrated slightly faster than uH2A on SDS gels (e.g., slightly faster than cuH2A in Figure 4). This ubiquitinated peptide was not observed. At more extensive digestion of trout liver H2A species (Figure 5D) there appears to be a minor population of ubiquitinated H2A molecules (labeled uP, u_2P, etc.) with slightly greater mobility when compared to the mobility of ubiquitinated H2B species. These may correspond to ubiquitin attached to the N-terminal portion of H2A (residues 1–114), or these fragments may be the result of other proteolytic activities due to the prolonged time required for digestion and greater amount of enzyme preparation added. It should be noted that the signal intensity for all ubiquitinated H2A species as well as H2A.Z was decreased at the most extensive digestion, which supports the idea of other proteolytic activities.

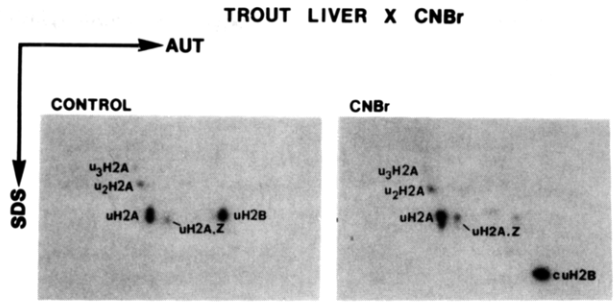

FIGURE 6: Identification of ubiquitinated peptides following cleavage of trout liver histones by CNBr. Trout liver histones were cleaved with CNBr and the resulting peptides analyzed as described in Figure 4. The ubiquitin-containing portion of the cleaved uH2B is designated cuH2B.

Cyanogen Bromide Does Not Cleave the Polyubiquitinated Histone H2A Species. In Figure 1 three possible arrangements are shown for a chain of ubiquitin molecules attached to one site on the histone. Model B shows ubiquitin molecules linked in a head-to-tail arrangement, with the C-terminal glycine of one ubiquitin linked to the N-terminal methionine of the next ubiquitin. The last ubiquitin moiety is linked via its C-terminal glycine to the ε-amino group of lysine 119 of H2A. In models C and D ubiquitin molecules are attached via isopeptide bonds to the ubiquitin covalently bound to the histone. If model B is correct, then CNBr cleavage of the Met residue at the N-terminus of ubiquitin would result in the disappearance of the polyubiquitinated histone H2A species and an increase in the amount of monoubiquitinated H2A. Neither ubiquitin nor trout histone H2A contains internal methionine residues (Watson et al., 1978; Connor et al., 1984). CNBr cleavage of trout liver histones does not result in removal of ubiquitin moieties from the polyubiquitinated H2A (Figure 6). Trout histone H2B contains methionine residues at positions 57 and 60 (Kootstra & Bailey, 1978) and is cleaved by CNBr. The ubiquitin-containing fragment, cuH2B, probably corresponds to the C-terminal peptide of H2B with one ubiquitin molecule attached.

Discussion

Rill and Oosterhof (1981) demonstrated that V8 protease could be used as a probe for exposed histone surfaces in nucleosomes. They found the protease sensitivities of the nucleosomal histones in chicken erythrocyte nucleosomes to be H3 ≫ H2B > H2A, H4. We found the relative susceptibilities of the histones in trout liver nucleosomes to be H2A ≫ H2B, H3, H4. Trout H2A is likely cleaved on the carboxyl side of Glu 121, which in chicken H2A is an Asp residue (Wu et al., 1986). Since V8 protease digests only Glu residues under the conditions employed, the carboxyl-terminal portion of chicken H2A would not be digested by this enzyme. The observation that the carboxyl-terminal portion of H2A in nucleosomes is accessible to proteolytic attack is in agreement with the studies of others (Hatch et al., 1983; Cary et al., 1978; Rosenberg et al., 1986).

Digestion of polyubiquitinated H2A by V8 protease and H2A-specific protease shows that a seven-residue fragment of H2A (residues 115–121), with lysine residues at positions 118 and 119, can contain one or more ubiquitin molecules. Similar results were obtained when calf thymus histones were used in similar experiments (Nickel and Davie, unpublished results). After incubation with V8 protease, uH2A in nucleosomes is processed to cuH2A, yielding a new doublet on the two-dimensional gel pattern (see Figure 3). Moreover, V8 protease treatment resulted in a shift of the mobility of the

polyubiquitinated histone H2A species to faster migrating forms, corresponding to the N-terminal H2A peptide (1–121) with one or more ubiquitin molecules attached. Smaller ubiquitinated peptides (e.g., a 9.1 kDa ubiquitinated peptide, which would correspond to ubiquitin attached to residues 122–127) were not observed. Digestion of trout liver histone H2A with histone H2A specific protease generates two peptides, a long N-terminal fragment (1–114) and a short C-terminal fragment (115–127). One or more ubiquitin molecules were attached to the C-terminal peptide. It is not entirely clear whether additional ubiquitin molecules can be attached to the N-terminal portion of the histone H2A molecule between residues 1 and 114. The results from these experiments indicate that the adduct resulting from such a linkage would be a minor component if found at all.

The experiments with cyanogen bromide demonstrate that in the ubiquitin chain ubiquitin molecules are covalently linked via isopeptide bonds (Figure 1, model C and/or D). In ubiquitin, lysine 6 was the most readily acetylated (Jabusch & Deutsch, 1985; Zhu et al., 1986) as well as fully exposed on the surface of the ubiquitin molecule (Vijay-Kumar et al., 1987). Thus, lysine 6 may be the site of ubiquitination.

Acknowledgments

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Registry No. Ubiquitin, 60267-61-0; L-lysine, 56-87-1.

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